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ORIGINAL RESEARCH ARTICLE

Campylobacter growth rates in four different matrices: broiler caecal material, live birds, Bolton broth, and brain heart infusion broth

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Background: The objective of this study was to characterise *Campylobacter* growth in enrichment broths (Bolton broth, brain heart infusion broth), caecal material (*in vitro*), and in the naturally infected live broilers (*in vivo*) in terms of mean lag periods and generation times as well as maximum growth rates and population (cell concentration) achieved.
Methods: Bolton and brain heart infusion broths and recovered caecal material were inoculated with 10 poultry strains of *Campylobacter* (eight *Campylobacter jejuni* and two *Campylobacter coli*), incubated under microaerobic conditions, and *Campylobacter* concentrations determined periodically using the ISO 10272:2006 method. Caeca from 10 flocks, infected at first thinning, were used to characterise *Campylobacter* growth in the live birds. Mean generation times (G) (early lag to exponential phase) were calculated using the formula: $G = t/3.3 \log b/B$. Mean lag times and μmax were calculated using the Micro Fit© Software (Version 1.0, Institute of Food Research). Statistical comparison was performed using GENSTAT ver. 14.1 (VSN International Ltd., Hemel, Hempstead, UK).
Results: The mean lag periods in Bolton broth, brain heart infusion broth, caecal material, and in the live bird were estimated to be 6.6, 6.7, 12.6, and 31.3 h, respectively. The corresponding mean generation times were 2.1, 2.2, 3.1, and 6.7 h, respectively; maximum growth rates were 0.7, 0.8, 0.4, and 2 generations h^{-1} and the maximum populations obtained in each matrix were 9.6, 9.9, 7.8, and 7.4 $\log_{10}$ CFU/g, respectively.
Conclusion: This study provides data on the growth of *Campylobacter* in a range of laboratory media, caecal contents, and in broilers which may be used to develop predictive models and/or inform science-based control strategies such as the maximum time between flock testing and slaughter, logistical slaughter, and single-stage depopulation of broiler units.

Keywords: Campylobacter; *Growth rates* in vivo *and* in vitro; *flock thinning*; *foodborne pathogen*

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As human campylobacteriosis is one of the most common foodborne diseases worldwide, *Campylobacter* have been extensively studied (1). This research has clearly identified poultry as the most important source with 50–80% of human *Campylobacter* cases attributed to the poultry reservoir as a whole (2). The European Food Safety Authority (EFSA) recently published a baseline study of *Campylobacter* prevalence in chickens within EU member states (3, 4). Most countries reported high prevalence of *Campylobacter* in broiler flocks, including Ireland where 83.1% of flocks were positive. Thus, many studies have focused on *Campylobacter* control in broilers, especially during primary production and processing. On-farm biosecurity and testing flocks to inform preventative measures such as logistic slaughter (slaughtering *Campylobacter*-free flocks before contaminated birds) are still the primary control activities. However, the effectiveness of this strategy depends on understanding the growth characteristics of *Campylobacter* (5). While it is known that Campylobacters are well adapted to survive and grow in the avian gut, and once introduced spread rapidly within

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flocks (6, 7), data on growth within the birds is scarce (5). Moreover, while research has identified the caeca, paired finger-like projections from the proximal colon at its junction with the small intestine, as the primary source of infection within birds (8), information on *Campylobacter* growth in caecal material and how this compares with growth in laboratory media is lacking.

Such data are important. For example, if *Campylobacter* grow rapidly in the birds, testing 4 days pre-slaughter (the time required to obtain a culture-based result) might result in false negatives and heavily contaminated flocks cross-contaminating birds slaughtered later in the day. Furthermore, predictive models, such as ComBase, used to estimate *Campylobacter* growth and model scenarios, such as the likely *Campylobacter* carriage levels if the birds are infected during first thinning, are based on broth cultures and comparative data on growth in caecal contents and the live birds is required to validate these models.

Such research is also timely, as the European Commission has recently published draft legislation amending Regulation (EC) No. 2073/2005 and proposing processing hygiene criteria (PHC) for the poultry sector. Under this legislation, 10 g of neck flap from 15 randomly selected birds per flock will be pooled to give 5×25 g final samples. Within a moving window of 50 samples, no more than 5 may exceed the limit of 10^3 CFU/g (9). As there is a direct relationship between the caecal concentration of *Campylobacter* and carcass contamination levels (10), each processor will have to decide if specific interventions are required to achieve this target based on pre-slaughter flock test data and a prediction of the likely increase in *Campylobacter* caecal concentration in the intervening period between testing and slaughter.

The objective of this study was therefore to provide the *Campylobacter* growth data required, including estimating mean lag periods and generation times as well as maximum growth rates and population (cell concentration) achieved.

Methods and materials

Inoculation study – Campylobacter *isolates*

Challenge studies were undertaken to investigate and compare *Campylobacter* growth in laboratory-based broths and caecal material. Poultry isolates (10) (eight *Campylobacter jejuni*, two *Campylobacter coli*) were used in the study (Table 1). Of these, six were obtained from the culture collection at Teagasc Food Research Centre (Ashtown) and four from the Animal Health Veterinary Laboratory in Surrey, UK.

Preparation of caecal material

Two thousand caeca from a random selection of flocks were collected by staff at the broiler processing plant between January and February 2014, immediately following slaughter and evisceration. Samples were delivered to the laboratory in Teagasc Food Research Centre, Ashtown, on the same day and processed within 24 h. Briefly, the contents were removed aseptically and pooled to create sample sets of 10×200 g and sent to the Agri-Food & Biosciences Institute (AFBI, Belfast) for irradiation (high dose 10 kGy). After irradiation, caecal contents were divided into 100 g portions and tested for *Campylobacter* using both direct plated and enrichment methods, according to the International Standards Organization Horizontal Method for Detection and Enumeration of *Campylobacter* spp. (11, 12). Briefly, 1 g of caecal material was added to 9 ml of Bolton broth (CM983B, Oxoid, Cambridge, UK) supplemented with 5% lysed horse blood and a selective supplement containing cefoperazone, vancomycin, trimethoprim, and cycloheximide (SR183E, Bolton broth supplement, Oxoid, Cambridge, UK) and vortexed. Serial dilutions were prepared in Maximum Recovery Diluent (CM0733B Oxoid, Cambridge, UK), and 100 μL volumes were plated out on modified *Campylobacter* blood-free selective agar (mCCDA, CM0739b, Oxoid, Cambridge, UK) supplemented with

Table 1. The strains used to inoculate caecal contents

Strain identity	Isolated from	Species and sequence type/clonal complex (when available)
CJ1	Broiler Farm, UK	*Campylobacter jejuni*
CJ2	Broiler Farm, UK	*Campylobacter jejuni*
CC1	Broiler Farm, UK	*Campylobacter coli*
CC2	Broiler Farm, UK	*Campylobacter coli*
LK115	Broiler Farm, Ireland – Caeca	*Campylobacter jejuni*, ST814/cc-661
ST45	Caeca, Ireland	*Campylobacter jejuni*, ST45/cc-45
LK016	Caeca, Ireland	*Campylobacter jejuni,* ST257/cc-257
LK014	Caeca, Ireland	*Campylobacter jejuni,*ST6764/cc-257
LK253	Caeca, Ireland	*Campylobacter jejuni,* ST6763/cc-661
11168	Human clinical strain	*Campylobacter jejuni* typed, national collection

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cefoperazone and amphotericin (SR0155E, CCDA selective supplement, Oxoid, Cambridge, UK). The remaining broths containing caecal contents were enriched by incubating under microaerobic conditions using Anaero Jars (AG0025A, Fannin, Dublin) with atmosphere generation Kits, Campygen (CN025A, Oxoid, Cambridge, UK) at 37°C for 5 h followed by 42°C for 48 h. After incubation, samples were plated out on mCCDA. All plates were examined to ensure absence of *Campylobacter* in the irradiated samples.

Preparation of inocula

Cultures were prepared from frozen stocks by aseptically placing one bead (TSC, Lancashire, UK) of each isolate in 25 ml Hunts broth containing 0.65 g nutrient broth (CM0001B, Oxoid, Cambridge, UK) and 0.15 g Yeast extract (CM0019B, Oxoid, Cambridge, UK), 5% Lysed Horse Blood (SR048C, Lennox, Dublin), and 0.4% *Campylobacter* growth supplement (SR0232E, Oxoid, Cambridge, UK). The inoculated broths were incubated under microaerobic conditions at 42°C for 48 h. After incubation, broths were vortexed for 30 s followed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was suspended in 25 ml phosphate-buffered saline (P4417, Sigma-Aldrich Arklow, Wicklow, Ireland) and vortexed. Cell suspensions were diluted to 10^{-3} in 9 ml Maximum Recovery Diluent (MRD, CM0733B Oxoid, Cambridge, UK). A 1 ml volume of the suspensions were then transferred to 99 ml Hunts broth to give a final cell concentration of 3 $\log_{10}$ CFU/ml for spiking of the caeca. Plate counts were carried to confirm spiking concentrations.

Inoculation of broiler caecal material and broths

Separate sterilised caecal samples (100 g) were then inoculated with 1 ml of each *Campylobacter* isolate and mixed thoroughly to give final concentrations of approximately 1 $\log_{10}$ CFU/g. Plate counts were performed to confirm and samples were then incubated at 42°C under microaerophilic conditions.

Preparation of inocula for broths

For broth inoculation, the above procedure for the preparation of inocula for caeca was followed with just a dilution difference; the broths were diluted five times to 10^{-5} in 9 ml MRD and then transferred to 99 ml of either Bolton broth (CM0983, Oxoid, Cambridge, UK) or brain heart infusion broth (CM1135B, Oxoid, Cambridge, UK) to provide an inoculum with approximately 1 $\log_{10}$ CFU/ml. Plate counts were performed to confirm this concentration.

Sampling time points

Each isolate was tested at the following times: (h): 0, 3, 6, 9, 15, 18, 21, 24, 27, 30, 33, 39, 42, 45, 48, 51, 54, 57, 63, 69, 72, 75, 78, 81, 84, 87, 90, and 93 through the use of alternating caecal samples. For example; caecal sample 1 was inoculated with the relevant isolate at 9 am and tested at the following times: (h): 0, 3, 6, 9, 24, 27, 30, 33, 48, 51, 54, 57, 72, 75, 78, and 81. Caecal sample 2 was then inoculated with the same isolate at 6 pm and tested: (h) 0, 15, 18, 21, 24, 39, 42, 45, 48, 63, 66, 69, 72, 84, 87, 90, and 93. All isolates were tested in duplicate and repeated on three separate occasions.

At each time point, 1 g or 1 ml of sample was placed in 9 ml MRD, vortexed for 30 s, diluted, and plated out on mCCDA, as previously described.

Farm Sampling – Sample Collection

Broiler farms (8) were sampled for this study between February and August 2014 to determine the *Campylobacter* growth profile in naturally contaminated broilers. The farms tested had flock sizes ranging from 25,000 to 33,000 birds with farm size on each site varying from one to six houses. A total of 10 caecal samples were aseptically collected from 15 flocks on these farms on day 28, at first thinning (partial depopulation at 35 days) and at time of final depopulation (final thinning at 42 days). On day 28, the caeca were aseptically removed by the company's veterinarian. At first and final thinning, caeca were collected from the slaughter plant immediately following evisceration. All samples were transported immediately in a cool box at approximately 2°C to the laboratory and analysed within 24 h.

Isolation of Campylobacter *spp. from caeca*

Samples were both direct plated and enriched as described previously. Briefly, 1 g of caecal material was added to 9 ml of Bolton broth and vortexed. Serial dilutions were prepared in MRD, and 100 µL volumes were plated out on mCCDA. The remaining broths containing caecal contents were enriched by incubating under microaerobic conditions as above at 37°C for 5 h followed by 42°C for 48 h. After incubation, samples were plated out on mCCDA.

Campylobacter *Identification*

All presumptive *Campylobacter* isolates were confirmed initially by Gram staining (3% w/v KOH, Sigma-Aldrich, Arklow, Wicklow, Ireland) and a series of biochemical tests (Oxidase test) (Oxoid, Cambridge, UK) and the L-ALA test (Sigma-Aldrich, Arklow, Co. Wicklow, Ireland) followed by streaking on Campy Food ID chromogenic agar (Biomerieux, Durham, NC).

Statistical Analysis

Campylobacter counts in all matrices were converted to $\log_{10}$ CFU/g. Generation times (G) (early lag to exponential phase) were calculated using the formula: G = t/3.3 logb/B, where t = time interval in h, b = number of bacteria at the end of the time interval, and B = number of bacteria at the beginning of the time interval (5). Lag times and μmax were calculated using the Micro Fit© Software (Version 1.0, Institute of Food Research) and graphs from this software used to calculate stationary, exponential, and decline phase information. Micro Fit© is a 32-bit application which is designed to give a graphical representation of microbiological data and fit a growth model to the data to obtain parameters (13). Statistical comparison of all parameters was performed in GENSTAT by Anova ver. 14.1 (VSN International Ltd., Hemel, Hempstead, UK) by comparing strain, medium, and interaction of strain and medium. Significance was determined at the 5% ($P < 0.05$ level).

Results

The mean lag period, generation time, maximum growth rate, and maximum concentration obtained in Bolton broth were 6.6, 2.1, 0.7 generations h^{-1}, and 9.6 $\log_{10}$ CFU/ml, respectively (Table 2). Statistically similar ($P > 0.05$) values were obtained in brain heart infusion broth at 6.7, 2.2, 0.8 generations h^{-1}, and 9.9 $\log_{10}$ CFU/ml, respectively. In contrast, the mean lag period and generation times in caecal contents (*in vitro*) were significantly ($P < 0.05$) longer at 12.6 and 3.1 h, respectively. The mean lag period and generation time were also significantly ($P < 0.05$) longer (31.3 and 6.7 h, respectively) in the broilers (*in vivo*) when compared with the other growth media (also Table 2). The maximum growth rate and concentrations achieved in caecal contents were 0.4 generations h^{-1} and 7.8 $\log_{10}$ CFU/g, respectively. The corresponding figures for *Campylobacter* growth in the broilers were 2.0 generations h^{-1} and 7.4 $\log_{10}$ CFU/g, respectively. While the former was statistically significant ($P < 0.05$), the latter was not. No significant differences were observed between the two species, *C. jejuni* and *C. coli*, in the inoculated broths or caecal material.

Discussion

This study observed mean generation times of 2.1 (126 min) and 2.2 h (132 min) in Bolton and brain heart infusion broths incubated at 42°C, respectively, over a period of time covering the early lag and exponential phases of growth. The mean μmax (exponential phase) were 0.7 and 0.8 generations h^{-1}, respectively. These are similar to the values reported previously (14) for *C. jejuni* F38011 (0.7 generations h^{-1}) and *C. jejuni* 02-833L (0.5 generations h^{-1}) in Muller-Hinton broth incubated at 37°C, and for *C. jejuni* (NCTC 11168) grown in Brucella broth; 0.61 generations h^{-1} at 37°C and 0.72 generations h^{-1} at 42°C (15). Interestingly, the mean generation time observed in broilers, 6.7 h, was also similar to the 4.9 h reported by a previous Irish study (5), although the study design (enumerating *Campylobacter* counts at first and second thinning and dividing by the time period in between) was very different.

Campylobacter grew well in the irradiated caecal materials in this study, achieving a mean generation time of 3.1 h, a max of 0.4 generation h^{-1}, and a maximum concentration of 7.8 $\log_{10}$ CFU/g. The latter is similar to the 7.8–8.2 $\log_{10}$ CFU/g reported (16) in the caeca of French broilers and the 8 $\log_{10}$ CFU/g obtained in Swedish birds (17) but considerably higher than the 5.5–6.6 $\log_{10}$ CFU/g found in Irish broilers (5). High caecal numbers were not unexpected as it is well established that *Campylobacter* grow well in caecal material (17–19), not least because of the microaerobic environment, nutrient availability, and the pH, typically 6.8 (20). Several studies have reported a positive correlation between the *Campylobacter* concentration in the caeca and mean carcass counts (10, 21). Thus, as the

Table 2. Growth parameters in Bolton broth, brain heart infusion broth, irradiated caecal contents, and in the birds (*in vivo*)

Matrix	Initial concentration ($\log_{10}$ CFU/g)	Lag period (h)		Generation time[1] (h)		μmax (generations h^{-1})		Maximum concentration observed	
		Mean	Range	Mean	Range	Mean	Range	Mean	Range
Bolton broth	1.0	6.6[a2]	2.3–9.0	2.1[a]	1.0–3.5	0.7[a]	0.1–1.1	9.6[a] $\log_{10}$ CFU/ml	7.5–10.7 CFU/ml
Brain Heart Infusion broth	1.0	6.7[a]	3.1–8.6	2.2[a]	1.0–3.6	0.8[a]	0.3–4.4	9.9[a] $\log_{10}$ CFU/ml	6.2–10.8 CFU/ml
Caecal (*in vitro*)	1.0	12.6[b]	6.5–20.5	3.1[b]	1.2–5.1	0.4[b]	0.1–0.9	7.8[b] $\log_{10}$ CFU/g	5.3–9.9 CFU/ml
Broilers (*in vivo*)	Unknown	31.3[c]	NA	6.7[c]	NA	2.0[c]	NA	7.4[b] $\log_{10}$ CFU/g	NA

NA = not applicable.

[1]Generation time (early lag and exponential phase).

[2]Numbers with the same superscript letter are not significantly different ($p < 0.05$).

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European Commission introduces PHC for the poultry sector based on *Campylobacter* skin flap counts, data on caecal concentrations will become an important resource for predicting the likelihood of achieving compliance. Interestingly, all studies reported *Campylobacter* caecal concentrations above 5 $\log_{10}$ CFU/g, the count at which the associated carcasses should be considered to be high risk (22, 23).

The maximum concentration of *Campylobacter* achieved in the laboratory broths (9.6–9.9 $\log_{10}$ CFU/ml) were significantly higher than those observed in the caecal inoculation studies (7.8 $\log_{10}$ CFU/g) and in the birds (7.4 $\log_{10}$ CFU/g). Moreover, the mean generation times and mean μmax in the broths were significantly ($P < 0.05$) different to those observed in caecal material and in the broilers. The differences between the laboratory broth and caecal/bird *Campylobacter* growth parameters may be attributed to the optimised growth conditions in the former and, at least in the case of the *in vivo* studies, the lack of competing microflora (24). Regardless, these differences call into question the appropriateness of using broth-based models for estimating *Campylobacter* growth in poultry.

It was concluded that *Campylobacter* grow well *in vitro* (broths and in caecal material) and *in vivo*, reaching concentrations in excess of 7 $\log_{10}$ CFU/g. However, the significant differences between key growth parameters suggest new models are required if a predictive approach is to be applied to inform the need for risk management practices such as logistic slaughter to achieve compliance with the proposed European Commission *Campylobacter* criteria. Moreover, the data provided in this study will contribute to the development of such predictive tools.

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Conflict of interest and funding

No conflict of interest declared.

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